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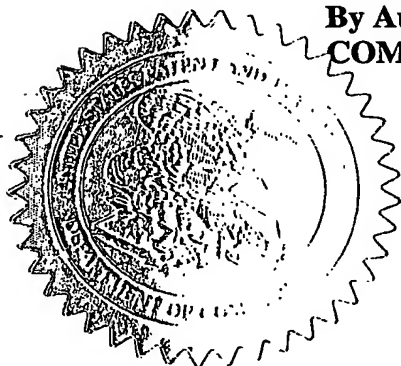
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# **PROVISIONAL APPLICATION FOR PATENT COVER SHEET** This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto							
TITLE OF THE INVENTION (280 characters max)							
Combinatorial Surface Chip Compositions That Allow Selection, Differentiation and Propagation of Stem Cells							
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Respectfully submitted,

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## **USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

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**PROVISIONAL APPLICATION**

**FOR**

**UNITED STATES LETTERS PATENT**

**BY**

**ZORINA GALIS AND J. CARSON MEREDITH**

**FOR**

**COMBINATORIAL SURFACE CHIP COMPOSITIONS THAT ALLOW  
SELECTION, DIFFERENTIATION AND PROPAGATION OF STEM CELLS**



# Combinatorial Surface Chip Compositions That Allow Selection, Differentiation and Propagation of Stem Cells

## Background of the Invention

5       The current procedures employing the therapeutic potential of stem cells continue to remain more an "art", which poses great problems with reproducibility and does not allow the reliability necessary for clinical applications. Thus, the precise design and standardization of conditions used for isolation, expansion, and  
10   differentiation of stem cells is desired. It is an object of the invention to provide methods and strategies for engineering stem cell biology. The term "stem cells" as used herein, refers to both committed and uncommitted stem cells.

      It is another object of the invention to provide approaches for  
15   expanding endothelial progenitor cells (EPC) *in vitro*. Currently available technologies for *in vitro* expansion of human stem cells (HSC) can be divided into stroma-based and stroma-free methodologies. Stroma-based protocols involve culturing HSC on "feeder layers" derived from bone marrow stromal cells. From a  
20   clinical standpoint, this approach may be unsuitable for expansion of peripheral blood stem cells (PBSC) for autologous transplantation, due to issues of cross-contamination and immune activation when PBSC from one donor are cultured on stromal cells derived from an unrelated human source.

25       A growing literature indicates that the adhesion, migration, proliferation, and differentiation of numerous cell types can be modulated by the underlying substrate properties, including surface chemistry, microstructure, and roughness. These differences in cell function are mediated by alterations in adhesive interactions  
30   primarily involving integrin receptors. Therefore, another object of

the invention is to provide methods for directing EPC function by controlling adhesive interactions through the underlying substrate chemistry, microstructure, and roughness. Previous analyses have demonstrated that adhesive interactions play important roles in bone marrow-derived stem cell proliferation and differentiation. The bone marrow presents a complex microenvironment in which the growth, differentiation, and self-propagation of hematopoietic stem cells are regulated by soluble and matrix-associated cytokines and by adhesive interactions with accessory stromal cells and the underlying extracellular matrix [113, 114]. Structural and functional compartmentalization of this microenvironment accounts for the amazing capacity of the bone marrow to generate a large diversity of mature cell types in response to the body's needs. Recent research indicates that interactions between hematopoietic progenitor cells and the supporting stroma are mediated by several families of adhesion molecules, including integrins, selectins, sialomucins, and members of the IgG superfamily [115, 116]. In particular, integrin-mediated adhesive interactions play important regulatory roles in hematopoiesis. For human hematopoietic progenitors, integrins  $\alpha 4\beta 1$  (VLA-4),  $\alpha 5\beta 1$  (VLA-5), and  $\alpha L\beta 2$  (LFA-1) are involved in homing of HSC to the bone marrow and adhesion to the stroma [117-124]. Moreover, integrin binding, in concert with hematopoietic growth factors, activates intracellular signaling pathways regulating hematopoietic progenitor cell survival, proliferation, and differentiation [117, 125-130]. In CD34<sup>+</sup> cells, for example, binding of integrins  $\alpha 4\beta 1$  and  $\alpha 5\beta 1$  to fibronectin in the presence of pharmacological concentrations of several cytokines promotes cell proliferation, while integrin engagement under low cytokine concentrations blocks cell cycle progression [131-133]. Although several integrin/growth factor-regulated signaling molecules, such as

PI-3 kinase, Src, and Ras/ERK members, appear to play key roles in integrin activation/adhesion and subsequent proliferative and differentiation events [134-142], the molecular mechanisms governing these responses remain poorly understood.

5           Given the complex interactions between substrate physical and chemical properties, integrins and other adhesion molecules, and signalling events, there is a distinct need for a novel "screening" technology for developing optimal substrate surfaces for stem cell expansion, survival, and differentiation. Thus, another object of the  
10       present invention is to provide a rational approach to engineer and discover surfaces that direct stem cell survival, expansion, and differentiation.

Multi-component combinatorial surfaces of varying physicochemistry and microstructure.

15           Thermally-controlled phase separation of biocompatible polymer blends (for example, but not limited to, poly(D,L-lactide), PDLA, and poly( $\epsilon$ -caprolactone), PCL) to generate surfaces with chemically distinct, heterogeneous microdomains enriched in one component are also disclosed. In studies with these substrates, the  
20       present methods have identified novel surface compositions that modulate cell adhesion, proliferation, and differentiation.

A specific focus of this invention is to utilize phase-separation and related phenomena to design surfaces optimized for obtaining endothelial cell progenitors to be used to treat patients with  
25       cardiovascular disease.

The endothelium plays a critical role in vascular homeostasis by secreting substances that not only acutely regulate vascular tone, platelet activity and coagulation factors, but also influence vascular inflammation, cell migration and proliferation over the longer term.  
30       Endothelial function may be determined *in vivo* by measuring the



vasomotor response to pharmacologic or physiologic stress. Subjects with coronary artery disease and those with risk factors have endothelial dysfunction, essentially secondary to reduced nitric oxide bioavailability [3]. Although its extent appears to correlate with the traditional risk factor "burden", there is considerable heterogeneity in the magnitude of endothelial dysfunction observed in individuals with similar risk factor profiles. Novel risk factors for atherosclerosis such as infections and genetic heterogeneity may partly account for this observed variability. More importantly, the presence and magnitude of coronary or peripheral endothelial dysfunction is an independent predictor of adverse long-term cardiovascular outlook even in patients without overt atherosclerosis. Thus, estimation of endothelial function provides a measure of vascular health or disease and can be used as a barometer of future risk of cardiovascular events.

Although much has been learned regarding risk factor-mediated injury to the vascular wall, little is known about mechanisms underlying repair. An examination of the relationship between the risk factor profile, endothelial dysfunction and circulating EPC activity with the hypothesis that the primary function of EPCs is to contribute to ongoing endothelial repair, is essential. Examination results demonstrated that EPC counts were lower in subjects with multiple risk factors and in those with endothelial dysfunction, with the worst dysfunction being present in those individuals with both risk factors and depressed EPC counts. This observation raises the possibility that the net vascular damage (endothelial dysfunction) results from a balance between endothelial injury from risk factor-induced oxidative stress, and repair by circulating EPC. A recent study has demonstrated that EPC activity and numbers can be stimulated significantly with statin therapy, raising the possibility that the improved endothelial function with

statins may at least partly be secondary to enhanced repair by EPC [8]. Thus, a determination as to whether proliferation of bone marrow-derived EPCs with colony stimulating factors results in improvement of endothelial dysfunction is required.

5        **Peripheral arterial disease (PAD) is a major public health problem associated with significant morbidity and mortality.**

10        Atherosclerotic PAD of the lower extremity, a condition that affects approximately 5% of the U.S. population over 50 years of age [9, 10], presents with intermittent claudication as the main symptom. Intermittent claudication is defined as pain in one or both legs that occurs with walking or exertion, does not resolve with continued activity, and abates (within 10 minutes) upon rest or reduction in  
15        walking pace. Symptoms are most frequently localized to the muscles of the calf and are manifested as alterations in resting hemodynamic measurements in the lower extremity [11]. With disease progression, unrelenting rest pain develops, eventually causing painful ischemic ulceration which can ultimately require amputation of the affected  
20        extremity. Smoking cessation, institution of anti-platelet therapy and statins represent important goals of treatment [12, 13]. In individuals with severe symptoms and identifiable proximal inflow disease, revascularization for aorto-iliac disease provides durable benefit [14]. In contrast, infra-inguinal disease, even if extensive, very rarely  
25        justifies surgical or percutaneous interventions, and treatment approaches are predominantly medical. The current therapeutic options available are primarily exercise, pentoxifylline, and cilostazol. Cilostazol, a Type III phosphodiesterase inhibitor, appears to improve peak walking time without biological modification of the underlying  
30        disease or significant incidence of side effects. Pentoxifylline provides only marginal benefits on walking ability and symptom relief. Thus,

the available options for treatment of patients with infra-inguinal disease are limited; therefore, these patients are excellent candidates for emerging therapies involving cell- or gene-based regimens. In addition, several factors render these patients ideal for testing  
 5 emerging therapies: a) easy access to afflicted tissue; b) simple delivery strategies that do not require sophisticated catheter-based technology; c) potential amplification of the angiogenic response secondary to ischemic upregulation of the receptor for vascular endothelial growth factor (flk-1) in hypoxic areas (especially with  
 10 recurrent ischemia such as in response to exercise); d) direct measurable end-points, such as walking distance on a treadmill, that serve as a direct surrogate for improvements in tissue perfusion and calf blood flow using non-invasive technology.

**Collateral formation involves cooperation of soluble  
 15 factors and stem cells.**

Although the events resulting in collateral development have not been fully elucidated, both angiogenesis and arteriogenesis are observed, processes that depend on the release and/or expression of a  
 20 number of growth factors. Angiogenesis refers to sprouting and proliferation of preexisting capillaries to form capillary networks, a process that relies on the dual stimulus triggered by hypoxia that promotes release of hypoxia inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF), and secondly, increasing shear  
 25 stress that leads to enlargement of pre-formed collaterals. In contrast, arteriogenesis does not require hypoxia, occurs in areas greatly proximal to ischemia, has enormous species diversity, and can take days or weeks to develop. Arteriogenesis is characterized by shear-mediated release of chemokines including macrophage  
 30 chemotactic factor(MCP)-1, intercellular adhesion molecule(ICAM)-1, and GM-CSF [15], that attract and stimulate circulating monocytes to

release TNF alpha and basic FGF, resulting in further attraction of monocytes, platelet activation, and release of growth factors.

Macrophages secrete proteases that participate in vessel remodeling, in endothelial and smooth muscle cell migration, and potentially in  
5 the transformation of capillaries into arterioles and conductance arteries [16].

Both experimental models and some human studies using either peptide or gene-transferred growth factors have shown some promise, but appear to be inadequate in promoting extensive  
10 collateralization [17-20]. It is unlikely that these factors are primarily responsible for arteriogenesis. Recent experimental data indicates that an important accompaniment of regional VEGF release during ischemia is the concomitant stimulation of bone marrow-derived EPC, with subsequent 'homing' of these cells to regions of  
15 ischemia and incorporation into the neovascularized area, but not other non-ischemic vascular beds [21].

There is now an understanding that stem cells have the capacity to differentiate down multiple pathways when introduced into a regenerating environment. The first evidence that this might  
20 occur came from studies in which whole bone marrow marked with a *LacZ* transgene was transplanted into lethally irradiated recipients and allowed to engraft and reconstitute the hematopoietic system. Two weeks after myocardial injury, donor-derived  $\beta$ -galactosidase-positive muscle cells were found to be incorporated into the  
25 regenerated myocardial tissue [22]. Cells from the bone marrow can differentiate into cardiac muscle, endothelial cells, osteoblasts, liver cell types, and neuronal and non-neuronal cells of the brain [23-31].

Strong evidence supports the bone marrow origin of cells participating in neovascularization.  
30

Studies show that endothelial cell progenitors reside in the bone marrow [32, 33], circulate [34, 35], and contribute to blood vessel formation during tissue repair and in pathological conditions, a process that closely resembles embryonic vasculogenesis [36].

5 Vascular smooth muscle cells and pericytes are also necessary components of neovascularization. Recent findings suggest that smooth muscle cell progenitor population is also present in the circulation, but probably in a lower frequency [37, 38], and other evidence indicates that endothelial progenitors also give rise to  
10 smooth muscle cell precursors [39].

There is increasing evidence that EPC primarily arise from hematopoietic stem cells (HSCs), although even this concept has come under recent challenge. Murine HSCs can be purified using a combination of cell surface markers such as the stem cell antigen,  
15 Sca-1 [40], the receptor tyrosine kinase c-Kit [41], and low or negative levels of lineage markers ( $lin^{-low}$ ) [42], or by using fluorescent vital dyes such as Hoechst 33342 [43]. Human HSCs have been isolated primarily through their expression of the marker CD34 [44], lack of lineage markers, and low expression levels of Thy1 [45]. However,  
20 there is also evidence for a CD34-negative hematopoietic stem cell [46, 47]. Finally, Jiang and colleagues have recently described a rare cell that can be isolated from the mesenchymal stem cell population in the bone marrow, muscle or brain, which was termed multipotent adult progenitor cell. This cell can be expanded for greater than 70 to  
25 150 population doublings and differentiates not only into mesenchymal lineage cells but also into endothelium, neuroectoderm, and endoderm [48-50].

EPCs have been detected in the periphery of infarcts, in hind limb ischemic models, in cutaneous wounds, in the corpus luteum,  
30 and in areas of tumor angiogenesis [51]. After administration of

VEGF, a 265% increase in monocytes and a 40% increase in lymphocytes occurred, whereas endothelial cell specific precursors were more substantially increased (CD34+ cells 355%, Flk-1+ 300%, VE-cadherin+ 155%) [52], and these cells homed into areas of

5 neovascularization. A similar increase in EPCs was observed in trials where VEGF was administered to patients [21]. In ischemic hind limb models, EPC mobilization is accelerated with a 2- to 5-fold increase in EPCs in peripheral blood observed in different species [53]. In

10 another study, EPCs injected into areas of myocardial ischemia accumulated in the ischemic area, incorporated into foci of myocardial neovascularization, and appeared to improve left ventricular function [33].

Compared to other cells, the magnitude of contribution of EPCs to vasculogenesis has been studied and estimated to range between 3

15 and 26% in different experimental settings [32, 33, 54, 55]. It appears that a more heterogeneous population, likely comprised of multipotent progenitors, tends to engraft to a higher degree into vessel structures [56]. Thus, strategies designed to stimulate a wide spectrum of progenitors, as proposed in this study, are likely to be

20 more successful. It appears that vascular endothelial growth factor (VEGF), by activating the tyrosine-kinase receptors VEGF1R and VEGF2R, acts as a key stimulus for EPC mobilization from the bone marrow. In humans, an early sharp rise in EPCs was noted after coronary bypass surgery (up to 50-fold increase), coinciding with a

25 VEGF peak which returned to baseline after 48 to 72 hours, demonstrating that dynamic mobilization can be stimulated by VEGF [57]. Humans have higher levels of EPCs in the circulation during the proliferative phase of the menstrual cycle and pregnancy. Moreover, recent studies have demonstrated that EPC activity and numbers can

30 be stimulated significantly with statin therapy [8, 58, 59]. Thus,

strategies that promote elaboration of bone marrow-derived progenitors with vasculogenic capabilities are worthy of investigation. Since the precise identification of a multipotent progenitor cell/s remains to be defined, a strategy that widely stimulates the bone marrow progenitor population is currently likely to provide the most effective and safe option.

**Endothelial Progenitor Cells (EPC) can be isolated from peripheral blood.**

The exact characterization of the EPC remains to be defined. Both hematopoietic and endothelial precursors express common epitopes that include Flk-1, Tie-2, CD34, Sca-1, c-Kit, thrombomodulin, GATA-4, GATA-6, and others [60, 61]. The expression of VEGF-2R or KDR receptor defines a subset of CD34/CD38 positive cells, some of which have the ability to differentiate along an endothelial lineage. AC133 (CD133) is a more primitive hematopoietic stem cell marker that is expressed on the majority of CD34+ cells, but unlike CD34, its expression is lost during maturation of EPCs, thus allowing an earlier and perhaps more precise identification of EPCs. However, AC133-negative cells and CD34-negative cells selected from peripheral blood, as well as the non-hematopoietic multipotent stem cell, will also form endothelial-like colonies and differentiate to produce cells expressing mature endothelial cell markers [62] [63]. Cell sorting is accomplished by flow cytometry after labeling peripheral blood samples for CD34, AC133, and KDR epitopes. Because of the limitations described above of cell sorting technologies in identifying EPCs within the mononuclear cell population, and the relative paucity of these cells (0.01%) in peripheral blood, functional assays have been developed. EPCs can now be isolated and reproducibly grown from peripheral blood samples or from buffy coats. In addition to cell sorting techniques, we

will therefore also measure peripheral blood EPC count by cell culture.

**Colony stimulating factors stimulate EPC mobilization and promote vascular growth:**

5

Given the potential of EPC to restore cardiovascular and tissue function, strategies aimed at enhancing the availability of such cells are necessary. Reports in the literature suggest that factors including cell density, cytokine concentrations, and surface chemistry influence the proliferation of EPCs *in vitro*. For example, collagen IV-coated surfaces promote differentiation of mouse embryonic stem cells to the endothelial lineage more effectively than do other surfaces including gelatin, fibronectin, and collagen I [64]. These data suggest that precise control of biomaterial surface properties can be utilized to encourage the selective attachment and growth of EPCs. However, methods for culture of EPCs *in vitro* currently constitute an art rather than a science – definitive conditions to ensure survival and expansion of EPCs to clinically useful volumes have yet to be established.

20

An alternative approach is *in vivo* manipulation of EPCs. Granulocyte-monocyte colony stimulating factor (GM-CSF) stimulates hematopoietic progenitor cells [65], myeloid lineage cells [66], and stromal cells [67], as well as augmenting EPC mobilization [68, 69]. Human endothelial cells have high-affinity receptors for GM-CSF, similar in number and affinity to those on myelo-monocytic cells [68-70]. Stimulation of these receptors transduces expression of c-fos followed by cell migration and proliferation [69, 71].

Takahashi and co-workers demonstrated that GM-CSF exerted a potent stimulatory effect on EPC mobilization, resulting in enhanced neovascularization of severely ischemic tissues as well as *de novo* vascularization of previously avascular sites. Using the rabbit

30



model of hind limb ischemia, they demonstrated that 7 days of daily GM-CSF (50 µg/day subcutaneously) augmented EPC-enriched cell populations (post-treatment vs. pre-treatment values  $\times 10^5/\text{ml}$ :  $12.5 \pm 0.8$  and  $6.7 \pm 0.3$ ,  $P < 0.01$ ) and EPC differentiation. Capillary density analysis showed extensive neovascularization, as well as improved ischemic/normal hind limb blood pressure ratio (with and without pretreatment:  $0.71 \pm 0.03$  and  $0.49 \pm 0.03$ ,  $P < 0.01$ ) [53]. The magnitude of effect of GM-CSF was not significantly different in this model from that observed with VEGF. To confirm that EPC proliferation is a key element in neovascularization, studies have demonstrated that *ex vivo* expanded EPCs, injected into a model of myocardial ischemia, produce improved neovascularization [72]. Improved revascularization of the ischemic mouse hind limb after treatment with G-CSF and GM-CSF is observed, compared to placebo. Hind limb ischemia was induced by ligation and excision of a segment of the proximal femoral artery. Animals were either treated with vehicle (saline), G-CSF or GM-CSF (the latter at  $10\mu\text{g/kg/day i.p.} \times 5$  days). After 7 days, the animals were sacrificed and perfused with a 5% barium sulfate/7% gelatin mixture. Following decalcification of the specimen, the hind limb arterial vasculature was imaged using high-resolution computed tomography (micro-CT). A marked and improved reconstitution of the arterial vasculature was observed in animals that received either G-CSF or GM-CSF. Fujita et al. showed that autologous bone marrow-seeded composite aortic grafts had rapidly accelerated surface endothelialization, by 80% at 1 week [73]. Similarly, grafts seeded with CD34<sup>+</sup> cells isolated from bone marrow have achieved early and enhanced endothelialization [74]. Shi and colleagues found that G-CSF treated grafts had significantly higher endothelialization than the controls, with extensive flow surface neointima covered with a single layer of

endothelium [75]. In addition, G-CSF therapy has been shown to mobilize primitive ( $\text{Lin}^- \text{c-kit}^+$ ) bone marrow cells that contribute to an improved blood supply to the ischemic myocardium and to restoration of function [76, 77].

5 Finally, in a recent study, 10 patients were treated in a placebo-controlled manner with 40  $\mu\text{g}$  of intracoronary GM-CSF followed by 2 weeks of alternate day subcutaneous injections of GM-CSF at 10  $\mu\text{g}/\text{kg}$ . Collateral flow was significantly enhanced [71]. However, mobilization of EPC was not investigated in this study.

10 **GM-CSF, G-CSF, and their combination mobilize hematopoietic stem cells in humans:**

GM-CSF, G-CSF and their combination are known to mobilize CD34+ cells from the bone marrow. Several studies have compared  
15 their relative efficacy for hematopoietic stem cell stimulation in healthy volunteers [78-80]. In general, G-CSF, as a single agent, appears to be superior to GM-CSF in mobilizing CD34+ cells and leukocytes. At a dose of 5 $\mu\text{g}/\text{kg}/\text{day}$  for 5 days, G-CSF caused a greater increase (37 fold increase, CD34+ cells = 29.5/ $\mu\text{L}$ ) above  
20 control versus GM-CSF (CD34+ cells = 8.0/ $\mu\text{L}$ ) [78]. Lane et al. compared G-CSF and GM-CSF at 10 $\mu\text{g}/\text{kg}/\text{day}$  and found values of 61/ $\mu\text{L}$  and 3/ $\mu\text{L}$ , respectively after 4 days [81]. However, GM-CSF appears to mobilize significantly greater numbers of the primitive CD34+/CD38-/HLA-DR+ subset of CD34+ cells when compared to G-  
25 CSF [80]. Finally, the concurrent administration of GM-CSF and G-CSF is associated with as good a yield of CD34+ cells as G-CSF alone, but with a greater yield of primitive CD34+ subsets [80, 81]. It is suspected that these primitive populations are rich in progenitor cells.

30 Side effects: G-CSF doses up to 24 $\mu\text{g}/\text{kg}/\text{d}$  have been given to donors [82, 83], but dose- escalation studies have shown that the

incidence and severity of side effects at doses  $>10\mu\text{g/kg/d}$  of either G-CSF or GM-CSF are increased out of proportion to any benefit in the form of mobilization of CD34+ cells [84, 85]. The Spanish National Donor Registry reported that the quantity of CD34+ cells collected  
 5 was related to the dose of G-CSF administered; when G-CSF was given for 5 days, more CD34+ cells were collected from people given  $10\mu\text{g/kg/day}$  ( $6.7 \pm 3.6 \times 10^6$ ) than from those given  $<10\mu\text{g/kg/day}$  ( $5.1 \pm 3.9 \times 10^6$ ). A dose of  $>10\mu\text{g/kg/day}$  ( $7.3 \pm 4.1 \times 10^6$ ) did not  
 10 significantly increase the yield. Side effects were more frequently reported in donors receiving  $>10\mu\text{g/kg/day}$  of G-CSF than in those receiving lower doses [86]. At present there is no information available regarding the potency of each of these cytokines, or their combination, in mobilizing EPCs in humans. What are needed are studies that are designed to determine which cytokine, or their  
 15 combination, is most efficient and safe at mobilizing EPCs.

**Matrix metalloproteinase (MMP)-9 is important for stem cell mobilization and survival.**

Results published in the last few months suggest that  
 20 production of MMP-9 in the bone marrow is essential in releasing the membrane form of Kit ligand (mKitL) to a soluble form (sKitL, also known as stem cell factor (SCF)) which conveys signals that modulate survival, adhesion, and motility of c-Kit+ HSC and endothelial cells. This action apparently enables endothelial and hematopoietic stem  
 25 cells to transfer from the quiescent to the proliferative niche [87]. The authors have also suggested that increased mobilization of stem cells after *in vivo* injection of GM-CSF is dependent on the upregulation of MMP-9 expression in the BM and its Kit ligand cleaving activity. There exists extensive expertise in the biology of MMPs, specifically  
 30 of MMP-9 in relation to vascular remodeling [16, 88, 89] and angiogenesis. Additionally, mice genetically engineered to be

deficient or transgenic for MMP-9 are readily available [88].  
Therefore, these tools are useful to investigate *in vitro* and *in vivo* the possibility that regulation of MMP-9 may be useful for control of stem cell mobilization and survival.

5           **Collateralization is heterogeneous in humans:**

          It is likely that the capacity for vasculogenesis and collateralization is variable among patients. This is often clinically evident in the presence of significant peripheral or coronary stenoses. Experimental evidence suggests that the capacity for collateralization  
10 is age-dependent, decreasing with advancing age [90-92].  
Furthermore, risk factors such as hypercholesterolemia and hypertension are also able to impede angiogenesis [93, 94]. Whether this is due to a defect in the ischemic substrate with aging and risk factors or to a defect in progenitor cell activity needs further  
15 investigation. Together with observations that EPC activity is lower in individuals with risk factors, the hypothesis that older patients with advanced atherosclerosis may have impaired ability to collateralize ischemic tissue gains validity. If this is true, then cytokine-induced mobilization of progenitors may provide  
20 replenishment of deficient or defective EPCs and promote angiogenesis. To investigate this further, one will have to study whether, compared to healthy subjects, patients with PAD have a lower potential to mobilize EPCs under resting conditions and/or after stimulation with colony stimulating factors.

## Brief Description of the Invention

Disclosed are methods and strategies for engineering EPC biology. Disclosed are methods for directing EPC survival, expansion, and differentiation by controlling adhesive interactions through the  
5 underlying substrates.

Stem cell adhesion, expansion, and commitment to differentiated phenotypes can be regulated through the chemistry and physical properties of the underlying substrate. The use of novel combinatorial "chips" containing thousands of surface features of  
10 varying chemistry, microstructure, and topography, created with phase-separation and related phenomena, are also disclosed herein. The term "stem cells" as used herein, refers to both committed and uncommitted stem cells. For example, uncommitted stem cells may be driven to differentiate based upon the chemical and physical  
15 properties of the above-described underlying substrate. Such differentiation may result in the production of committed stem cells.

The engineering of novel synthetic and hybrid substrates that allow the control of progenitor cell adhesion, maintenance, self-renewal, and differentiation, is disclosed. The *rationale* for this  
20 research is that the engineering of well-defined substrates that control stem cell expansion and differentiation will lead to robust stem cell-based strategies for the treatment of diseased tissues and organs. The frequency and function of these progenitor cell populations from the bone marrow and the circulating pool can be  
25 compared to establish potential differences in activities. The standardization of methods, as disclosed herein, for the *ex vivo* survival, expansion and differentiation of endothelial progenitor cells (EPC) isolated from animals (mice and pigs); to study the effects of adoptive transfer of cultured EPC in experimental models of ischemia  
30 in small animals and in pigs; and to translate promising leads from

these pre-clinical model systems into clinical studies in patients with vascular disease. Issues such as enhancing EPC numbers and functionality using *ex vivo* expansion and/or *in vivo* administration of colony stimulating factors will result in improvement of

5 vascularization and restoration of function in ischemic tissues is addressed herein. The mononuclear cell fractions from bone marrow specimens, and from peripheral blood before and after mobilization with colony stimulating factors, are studied *in vitro* to determine the best source for efficient isolation and expansion of EPC. Human

10 studies involve *in vitro* and *in vivo* analyses of circulating EPC in healthy subjects and in those with atherosclerosis, as well as clinical trials of colony stimulating factor therapy in patients with peripheral arterial disease.

#### Detailed Description of the Invention

15 Engineer substrates that control progenitor cell adhesion and that support their *in vitro* expansion and survival.

EPC survival and self-renewal can be modulated by controlling adhesive interactions through the underlying substrate. Multi-

20 component combinatorial surfaces (Combi-chips) of varying physicochemistry and microstructure are used to analyze the effects of a wide range of surface properties on EPC adhesion, survival, and proliferation. Progenitor cells are cultured on engineered substrates and adhesion, survival and proliferation is analyzed using specific biological markers of growth, proliferation, differentiation and anti-

25 apoptotic signals induced upon adhesion of purified stem cells. Stem-cell-preserving substrates will induce anti-apoptotic signals such as FAK activation, *bcl* family members, and PI3-kinase while stem cell proliferation will be associated with up-regulation of growth signals such as Myc and the Ras/ERK pathway. These experiments lead to

the identification of specific surface chemistries and formulations (composition/microstructure) that support enhanced stem cell adhesion, survival, proliferation, and preliminary EPC differentiation. The small subset (4-6 surfaces) of candidate

5 substrates can then be analyzed for separately for differentiation and in vivo functional outcomes. In parallel, evaluations of these candidate substrates are used to engineer second-generation surfaces for enhanced control of stem cell adhesion and function.

A specific application currently tested is to direct differentiation of

10 stem cells adhering to engineered substrates to lineage-specific endothelial progeny and test their use for the enhancement of *in vivo* therapeutic angiogenesis and collateral formation in experimental models. Substrates that induce growth signals in stem cells will be more compatible with stem cell differentiation, and that lineage

15 commitment will depend upon the presence of specific growth factors in the micro-environment. Stem cells collected from animals (e.g., genetically engineered mice, pigs), or from human bone marrow donors are investigated *ex vivo*. The ability of *ex vivo* generated animal endothelial cells to re-populate appropriate tissue

20 compartments will be tested in experimental animal models of human peripheral arterial disease (PAD), which involve angiogenesis and arteriogenesis. These *in vivo* animal studies allow one to address mechanistic questions pertinent to the use of EPC mobilization in clinical trials for treatment of PAD. For example, one is able to

25 investigate whether the functional effects of EPC mobilization result from formation of new capillary networks or from repair of existing, damaged vessels. Disclosed herein is a comparison of *ex vivo* expansion and differentiation of human EPCs from both normal and patients with atherosclerotic disease, collected before and after *in*

30 *vivo* mobilization with colony-stimulating factors. Peripheral blood

mononuclear cells is compared to those derived from bone marrow to determine which source is more suitable for efficient isolation and expansion of progenitor cells on surface-engineered substrates. Analysis of results are performed, and the conclusions are used as feedback for developing new surfaces for refining the remaining cell culture conditions (e.g., soluble additives) for the *in vitro* studies. *Ex vivo* expanded cells may be re-injected in patients to improve oxygenation in tissue rendered ischemic due to arterial obstruction (e.g., myocardial infarction, stroke) through enhanced formation of collaterals. Also, EPC may be used to promote regeneration of the endothelial lining of diseased arteries damaged by stroke-induced ischemia. The use of EPC expanded *ex vivo* is examined in pre-clinical studies using a pig model as a prelude to future interventional tissue-replacement trials. Testing the possibility to expand EPC *in vivo* by therapy with granulocyte monocyte colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and their combination. This has two main purposes. First, assess whether *in vivo* expansion modifies the properties of circulating progenitor cells (e.g., adhesiveness, survival, potential for differentiation). Second, assess whether the combination of the two expansion procedures has a synergistic effect upon the yield of desired progenitors and clinical benefit.

Conclusion from experimental and preclinical studies is tested for suitability for improvement in endothelial dysfunction and ischemia in patients with risk factors for atherosclerosis and those with peripheral arterial disease (PAD) in clinical trials. Endothelial dysfunction is secondary to a combination of exposure to injurious risk factors and deficient repair mechanisms, resulting from exhaustion or depletion of bone marrow-derived EPC. Estimates with regard to EPC activity in peripheral blood serve as a biological index



of bone marrow progenitor cell activity at rest and after cytokine stimulation. Measuring progenitor cell numbers using both an *in vitro* colony forming assay and quantitative flow-cytometric analysis of mononuclear cells expressing EPC epitopes (CD34, KDR, and AC133). The growth potential of EPC from untreated and treated healthy or atherosclerotic subjects will also be determined *in vitro* using described methodologies to develop a better assay of progenitor cell activity. Brachial arterial endothelial function and blood flow in the lower limbs of patients with PAD, can be used to assess the functional effect of EPC activity at rest and after stimulation. *Ex vivo* expansion of human EPCs using this method may in future prove to be especially useful in treatment of patients whose circulating EPC counts remain low even after CSF therapy.

Identification of novel surface formulations that control adhesion, survival, and proliferation of progenitor cells and promote differentiation of EPC has been accomplished. The capacity of *ex vivo* expansion of EPC to rescue vascular function in animal models has been established. Finally, an evaluation of the potential of human EPC to proliferate on the engineered substrates and the ability of cytokine-mobilized EPC to restore vascular function in patients with peripheral vascular disease has been determined. Specific information derived from such studies as described below permit the design of two-dimensional and/or three dimensional scaffolds for *in vivo* application. For example, once an optimal surface "formulation" is discovered combinatorially, the formulation can be used to fabricate laboratory- or clinical-scale two-dimensional surfaces for the in-vitro expansion and differentiation of stem cells in therapeutic quantities. In this context, a surface "formulation" would consist of the composition of components (polymers, proteins, polypeptides, minerals, and other nutrients) and the mixing and annealing

(thermal treatment) conditions. Likewise, three-dimensional porous scaffolds can also be fabricated for tissue engineering applications by integrating the surface formulation, discovered combinatorially, into the existing procedure for creating porosity. For example, the

5 porosity may be introduced via the common method of salt leaching, in which salt particles are dispersed into a 3D polymer sample prepared at the optimal formulation composition. Prior to leaching the salt to create pores, the sample would be thermally annealed to create the desired surface microstructure at the interface between the

10 polymer and salt. When the salt is leached, the scaffold contains pores of a desired size in which the surface of the pores expresses the optimal surface physical and chemical features. Such scaffolds may be useful, for example, in tissue engineering or bone regeneration. Collectively, these studies establish an integrated approach to

15 engineer controlled survival, expansion, and differentiation of stem cells for therapeutic applications. Similar approaches may be used in studies to control the availability and function of other essential progenitors, such as hematopoietic and neural stem cells.

#### 20 **Example I. Preparation of Combinatorial Biocompatible**

##### *Polymer Substrate Libraries*

Phase separated surface features and their effects on cell function were studied with combinatorial libraries for a blend of biodegradable PDLA (Alkermes, Medisorb 100DL,  $M_w=127000$ ,

25  $M_w/M_n=1.56$ ) and PCL (Aldrich,  $M_w=114,000$ ,  $M_w/M_n=1.43$ ). PDLA/PCL blends exhibit lower critical solution temperature (LCST) phase behavior, where PDLA and PCL separate at  $T > 86^\circ\text{C}$ . LCST phase transition allowed for the adjustment of microstructure and roughness via composition ( $\phi$ ), processing  $T$ , and processing time

30 (Figure 1 A-C). When the blend is quenched back to room

temperature, the two-phase structure is preserved due to the glass transition of PDLA (55°C) and crystallization of PCL ( $T < 60^\circ\text{C}$ ).

Figure 1 A-C presents optical and atomic force micrographs (AFM) of PDLA/PCL blends of various  $\phi_{\text{PCL}}$  and annealing  $T$  collected from a single  $T$ ,  $\phi_{\text{PCL}}$  combinatorial library. The feature size of PCL-rich domains (bright areas in Figure 1 A-C) varies from about 100 nm to over 100  $\mu\text{m}$  as a function of anneal  $T$  and  $\phi_{\text{PCL}}$ .

The primary surface differences between regions within and outside the LCST are the microstructure (lateral distribution of chemically distinct domains) and surface roughness. AFM and optical images from libraries were used to quantify the surface roughness and microstructure sizes. The diameter of PCL-rich regimes,  $d_{\text{PCL}}$ , increases with both  $f_{\text{PCL}}$  and  $T$  (Fig. 2A), and covers a range of ( $0.2 < d_{\text{PCL}} < 60$ )  $\mu\text{m}$ . Beyond  $d_{\text{PCL}} = 60$   $\mu\text{m}$  the PCL phase becomes continuous with dispersed PDLA droplets. Phase separation also induces changes in the roughness of the surface, the attributed to surface tension differences between chemically distinct domains and between crystalline and amorphous PCL. With a few exceptions root-mean-square (rms) surface roughness,  $R$ , increases with both  $f_{\text{PCL}}$  and  $T$  over a range of ( $2 < R < 500$  nm (Fig. 2B).

## Example II. Testing the Biological Effects of Adhesion to Combinatorial Surfaces on Stem Cells.

Mouse and human cells were used to determine the differential adhesive properties, of combinatorial surfaces, for stem cells and whether specific regions of combinatorial surfaces will enhance viability, proliferation and differentiation of these cells.

Mouse stem cells. Whole bone marrow was obtained by flushing the femurs and tibias of donor mice. Following RBC lysis, bone marrow mononuclear cells (MNC) will be isolated by Ficoll-Hypaque density gradient centrifugation. The pre-annealed  $T, f$

libraries were prepared and quenched to room temperature and  
sterilized in 70% ethanol in a laminar flow hood. Whole bone marrow  
or purified bone marrow MNC were cultured for 4-7 days directly on  
the surface of these in a -MEM supplemented with 10% FBS and 50  
5 ng/ml VEGF to promote survival and differentiation of endothelial  
progenitors. In some experiments, bone marrow cells were cultured  
with one or more additional cytokines (e.g. SCF, IL-3) to determine  
whether there was a synergistic effect between cytokine stimulation  
and polymer surface properties. Cells were cultured in parallel on  
10 glass coverslips and on tissue culture polystyrene as uniform surface  
control s for comparison to two-dimensional libraries. All cultures  
were performed with a minimum of triplicate repetition of each  
library.

Human stem cells. Leukapheresis products were obtained  
15 from patients under going stem cell mobilization and collection for  
transplant at Emory Univeristy Hospital. Following centrifugation of  
WBC suspensions on Ficoll-Hypaque to remove dead cells, CD 34+  
cells ere isolated by positive selection using immunomagnetic bead  
fractionation on a MiniMACS magnet system (Miltenyi Biotec).  
20 High-speed FACS sorting using a FACSVantage cell sorter further  
purified the CD34+ enriched fraction.

Positive selection of CD 34+ human HSCs was performed using  
immuno-magnetic bead fractionation. Human CD 34+ stem cells  
were then cultured on chips to investigate the effect of various  
25 surface properties upon cell adhesion and viability similar to the  
procedure used for mouse stem cells.

**Example III. The effects of pre-coating the combinatorial  
libraries with extracellular matrix (ECM) proteins including  
fibronectin, collagen IV, and laminin.**

30

Combinatorial polymer libraries were incubated with  $5\mu\text{g}/\text{cm}^2$  of ECM proteins for 1 hr at  $37^\circ\text{C}$ , then blocked with 1% BSA for 1 hr at  $37^\circ\text{C}$ . Comparison of pre-coated and uncoated libraries will provide an indication of whether microstructure affects cell function directly, or only secondarily through its influence on ECM protein conformation.

Cell staining and characterization. Total cell adhesion and viability were assessed by staining cultures with calcein AM and ethidium bromide for visualization by fluorescence microscopy (Live-Dead assay, Molecular Probes). For confirmation of EC identity and quantification of differential adhesion of ECs/EPCs and white blood cells (WBCs), double labeling immunocytochemistry was used for CD31 (EC marker) and CD 45 (WBC-specific marker) using secondary antibodies labeled with Rhodamine Red-X and Alexa Fluor 466, respectively.

To be able to directly test for differentiation towards the endothelial cell lineage, mice that express the endothelial specific promoter Tie-2 driving the expression of green fluorescent protein (GFP). These are also useful for tracking in vivo, after expansion in vitro on various surface-modified substrates for their ability to engraft in lethally irradiated recipient mice.

#### **Example IV. Analysis of Combinatorial Polymer Libraries.**

Fluorescence images were acquired on a 3 X 3 mm grid to cover the entire parameter space of the combinatorial library, so that cell density and viability could be correlated to polymer composition and material properties. Matlab software was used to generate a density contour map for the entire surface from this data. For initial rapid screening of polymer libraries, the adherent cells were stained with Cy5-labeled secondary antibodies and counterstained with SYBR Green I, then scanned with the 473 nm and 633 nm excitation

wavelengths on a Fuji FLA3000 phosphoimager. This approach permitted rapid visualization of the entire surface area.

Mouse bone marrow (BM) cells were cultured on combinatorial chips for up to four days. Adherent cells were stained using  
5 immunocytochemistry for CD 31 (EC-specific marker). Fluorescence images were acquired on a grid that covers the entire  $T, f$  space of the combinatorial library, so that cell density and viability can be correlated to polymer composition and material properties. These tests have demonstrated differential adhesion and viability of mouse  
10 BM cells cultured on polymer libraries. The differences in adherent cell morphology as a function of position suggest that BM cells belonging to different lineages adhere preferentially to areas having specific surface properties. These results illustrate the feasibility of the high-throughput method for culturing cells directly on  
15 combinatorial libraries. The effect of coating chips with natural extracellular substrates was also examined.

The effects upon differentiation towards the EC lineage by using bone marrow cells isolated from mice genetically engineered to express green fluorescent protein under the control of the Tie-2  
20 endothelial-specific promoter. An advantage to this approach is that one can monitor the differentiation of live cells (no additional processing that harms the cell is required), and track them in vivo (for example, to explore homing and integration of stem cells injected after controlled manipulation in vitro).

We claim:

1. A method of inducing stem cell adhesion, survival, proliferation, or differentiation comprising contacting a stem cell with a combinatorial surface.
- 5        2. The method of claim 1 wherein the combinatorial surface is coated with extracellular matrix.
3. The method of claim 1 wherein the combinatorial surface is coated with polymer.
4. The method of claim 1 wherein the stem cell  
10 differentiation is specific.
5. A combinatorial substrate library containing multiple variations of surface compositions, microstructures, and roughnesses, for inducing stem cell adhesion, survival, proliferation, or differentiation.
- 15        6. The combinatorial substrate library of claim 5, wherein the substrate is extracellular matrix.
7. The combinatorial substrate library of claim 5, wherein the substrate is polymer.
8. The substrates of claim 6 or claim 7, wherein the  
20 substrate is coated on a chip.
9. The combinatorial substrate library of claim 5, wherein the library is a chip library.
10. A combinatorial polymer substrate for inducing stem cell adhesion, survival, proliferation, or differentiation.
- 25        11. The combinatorial polymer substrate of claim 7, wherein the substrate is a chip coated with polymer.
12. The combinatorial polymer substrate of claim 7, wherein the substrate is a chip coated with extracellular matrix.

13. A polymer substrate or scaffold for inducing stem cell adhesion, survival, proliferation, or differentiation three dimensionally in vivo.

14. The polymer substrate or scaffold of claim 13 wherein  
5 the stem cell differentiates into tissue and induces further tissue  
formation in vivo.



### Abstract of the Invention

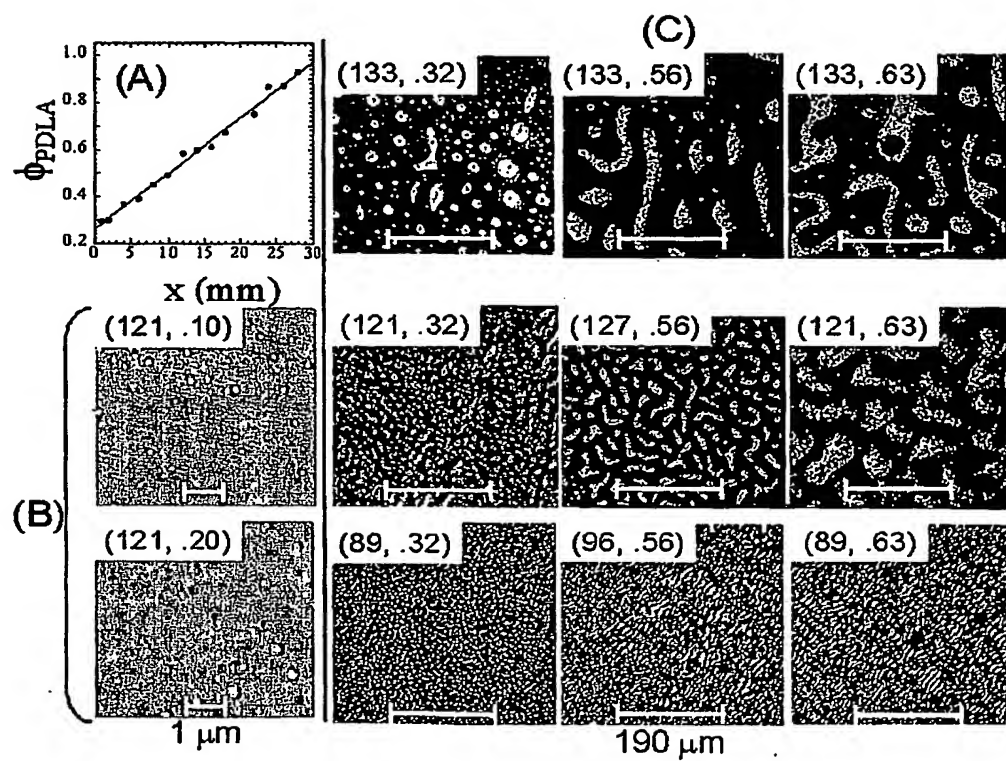
Methods and compositions relating to novel surface-modified substrates (chips) that facilitate the creation of a ready source of autologous hematopoietic and vascular repopulating stem cells for the

5 regeneration of the immune and circulatory systems of persons exposed to lethal and sublethal radiation injury. This technology precisely controls the chemistry and geometry of these surfaces to manipulate stem cells at will. Several different substrates have been developed, whose engineered properties will specifically enhance

10 survival, proliferation, or differentiation of cultured adult stem cells. Progenitor cells can be harvested from persons to produce individualized chips that are later used to restore essential body tissues and functions.

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Figure 1



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